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The Ets family of transcription factors are downstream targets of many signal transduction steps in tumor cells. Overexpression the DNA-binding domain of Ets2, which interferes with Ets-dependent transcription, inhibits Ras-mediated cellular transformation. We hypothesize that altering Ets activity might be an effective way to reverse the transformed phenotype of breast cancer cells. We analyzed the effect of stable expression of a variety of Ets2 constructs in RAS-transformed NIH-3T3 cells (DT-3T3) and in a human breast cancer cell line (MDA-MB-435). Overexpression of the dominant inhibitory mutant Ets2DBD lead to loss of anchorage independent growth in soft agar in both cell types. Unexpectedly, in DT-3T3 cells, high expression of Ets2 lead to reversion of transformation parameters like loss of anchorage independent growth, reorganization of stress fibers and reduced tumorigenicity in nude mice. An even stronger transcriptional activator, VP16Ets2 increased the apparent reversion activity in these cells. In MDA-MB-435 cells, high levels of Ets2 seem to be toxic to these cells, but moderate amounts can also revert these cells to anchorage dependence. These results suggest that the balance of Ets factors is important for maintaining multiple aspects of the transformed phenotype of breast tumor cells, and are an appropriate target for intervention.

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Gabriel Foos 01/27/00

PI - Signature Date

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Introduction and Background

Breast cancer is one of the major causes of death in women. Cancers arise as a result of multiple genetic aterations that eventually lead to a tumorigenic phenotype. One frequently found alteration is the constitutive activation of the Ras signaling pathway. Oncogenic Ras can cause widespread changes in cellular growth. To date, the majority of the downstream target genes of oncogenic Ras signaing that cause the transformed phenotype still remain unclear. Some of the targets that have been identified are transcription factors like the members of the Ets family. These transcription factors share a conserved DNA binding domain. Some members of this family are downstream targets of the Ras signal transduction pathway The transcriptional activity of the Elk family of Ets ternary complex factors are regulated by the Neu/Ras/Raf/MEK/MAP kinase pathway (1). We found that Ets1 and Ets2 are transcriptionally activated by Neu/ErbB-2 and Ras (2-4). Some Ets factors like ERF or Net are negative regulators of transcription. Their negative regulation can be relieved by stimulation of the Ras signaling pathway (5,6).

The Ets family and cellular transformation. It has been shown that members of the Ets transcription factor family are essential targets in cellular transformation in rodent fibroblasts. Most of the negative mutants that block Ras-mediated transformation also block activation of reporter genes that contain Ets and AP-1 sites. In rodent fibroblasts, we and others could show that dominant inhibitory mutants of Ets or AP-1 can block or even reverse Ras-mediated cellular transformation without interfering with normal cell growth (4,7,8). Since all of the Ets factors bind to the same consensus binding site, a dominant inhibitory mutant, consisting of the DNA binding domain alone, can act as broad inhibitors of Ets function. These data suggest, that the Ets transcription factors are essential for mediating the effects of more upstream, non-nuclear oncogenes.

Mutant Ets proteins can reverse the transformed phenotype of tumor cell lines. It has been reported, that a dominant inhibitory mutant for AP-1 can inhibit transformation of breast cancer and epidermal tumor cell lines (9). One study showed that overexpression of Ets1 in a human colon cancer cell line could reverse the transformed phenotype of this cells, without altering their attached growth properties (10). Another study showed that Ets2 plays a critical role in regulation of anchorage independent growth of BT20 breast cancer cells (11). These results indicate that altering Ets activity can indeed revert transformed human epithelial-derived cells. These findings encouraged me to investigate whether expression of Ets2 mutants can reverse the transformed phenotype of epithelial-derived breast tumor cell lines.

Ets activation and metastasis. The expression of many of the known metastasis associated genes is increased by activated Ras. Several of the promoters of these genes contain Ets and AP-1 binding sites (12), like the metalloproteinases collagenase, stromelysin (13,14), or the urokinase-type plasminogen activator uPA. Our lab could show that the uPA promoter can strongly be activated by Ets2 and Ras (3). The enahncer for keratin 18, a diagnostic marker for 90% of invasive breast carcinomas, also contains a Ras and Ets responsive element (15). I hypothesize that some Ets mutants can inhibit invasiveness *in vitro* and *in vivo.*

Objectives and Hypothesis

The study was designed to determine whether alteration of Ets activity, by expressing mutant forms of Ets2, will reverse the transformed phenotype of model cell lines and breast tumor cell lines. This analysis will give insight, whether the family of Ets transcription factors are novel downstream targets for therapeutic intervention in breast cancer.

The hypotheses are: 1) that Ets factors are downstream targets for signal transduction pathways in breast cancer cells and 2) that interfering on the level on Ets transcription factors, by overexpressing different mutant forms of Ets2, reverses the transformed phenotype and/or metastatic potential of breast cancer cell lines.

Progress: Considerable progress has been made towards objectives 1, 2, and 3.

Objective 1: Comparison of the effect of Ets2 mutants in reversing the transformed phenotype of rodent fibroblasts and human epithelial breast cells.

Milestone 1: Generation of stable Ets-expressing DT-3T3 cell lines. Several different Ets2 mutant constructs were generated to study the role of Ets proteins in Ras-mediated cellular transformation, including expression constructs for full-length Ets2, the Ets2 DNA binding domain (Ets2DBD) and the Ets2 transactivation domains (Ets2TAD), or VP16 Ets2, a construct that contains the VP16 transactivation domain fused to full-length Ets2. To analyze the effects of these constructs, I generated v-Ki-Ras-transformed NIH3T3 cells (DT-3T3 cells) (16), overexpressing the various constructs or the empty expression vector. I used the pCIN4 vector to improve the frequency of G418 resistant colonies (17). For the experiments described below, several individual clones were randomly picked, expanded and analyzed. At least 6 different clones per expressing construct were analyzed to exclude clonal variations (a total of approximately 100 clones). This very time consuming analysis was needed to identify the Ets2 construct that showed the highest reversion potential and forms a fundamental basis for all the objectives below.

Milestone2: Determination of the protein levels of Ets2 constructs in the reverted cell lines. All of the G418 resistant, expanded clones of full-length Ets2, the Ets2DBD, Ets2TAD and VP16Ets2, each tagged with the FLAG epitope, expressed a detectable amount of protein, when assayed by western blot, using an anti-Flag antibody. The VP16Ets2 construct was used to investigate, whether the addition of the VP16 transactivation domain to the transcriptional activator Ets2 can even further increase the reversion ability, indicating that Ets2 reverts these cell lines by active transcriptional activation.

Milestone 3: Expression of different Ets2 constructs leads to loss of anchorage independent growth of DT-3T3 cells. Soft agar assays have been used to analyze anchorage independent growth of DT-3T3 cells expressing the various Ets2 constructs or the empty expression vector pCIN. The number of soft agar colonies of the different individual clones were compared to the number of soft agar colonies that were formed by the control lines expressing the empty vector. Surprisingly 7/11 of the cell lines expressing the transcriptional activator Ets2 showed a greater than 4-fold reduction in soft agar growth when compared to the control lines. Most of the clones that express only the Ets2 Transactivation Domains (Ets2TAD) also showed reduced growth in soft agar. Finally, the overexpression of the Ets2 DNA binding domain also reduced soft agar growth significantly (fig. 1A). In parallel to all the soft agar assays, attached growth assays have been performed to see whether the reduced soft agar colony formation is due to impaired attached growth. The result showed clear reduction in anchorage-independent growth but not in attached growth (see fig. 1B). Also the growth curves for all the cell lines showed that they grow attached at the same rate during exponential growth, but consistent with the reverted phenotype, full-length or Ets2DBD expressing cell lines exhibited reduced saturation density. Comparison of the expression levels of the proteins in the individual lines, and the amount of colonies in the soft agar assay, showed that there was a direct correlation between the amount of protein and the apparent reversion of the individual lines.

Since DT-3T3 cells express endogenous Ets2, and to determine how much overexpression is needed to achieve reversion of the transformed phenotype, I performed RNAse protection assays. I estimated that a 10-fold overexpression of full-length Ets2 is needed to see the strong reduction in soft agar growth (not shown). I also tested the level of VP16Ets2 expression and found that they were 4-fold higher than for the highly reverted cell line Ets2full#5. Because all the VP16Ets2 clones had higher stable protein levels, I cannot exclude that the stronger reversion activity is a result of a more stable protein level in the VP16Ets2 clones. However, expression of VP16Ets2, a powerful transcriptional activator, clearly caused a reversion of Ras transformation.

Morphological reversion of DT-3T3 cells by over expression of full-length Ets2, the EtsDBD but not Ets2TAD. One characteristic of fibroblasts is their well organized actin filament structure. In contrast, the DT-3T3-cells show no organization at all. By staining the actin filaments with rhodamine conjugated phalloidin, I saw that the full-length Ets2 lines as well as the Ets2DBD lines, but not the Ets2DBD clones showed a nicely organized stress fiber structure. These findings were consistent with the morphological changes that I observed by examining the cells using phase-contrast. The full-length Ets2 lines and the Ets2DBD lines were non-refractile and flat like normal NIH3T3 cell, but in contrast, the Ets2TAD expressing lines were spindly and refractile like the parental DT-3T3 lines and the control lines containing the empty expression vector. These data suggest that there are differences in targets of these Ets2 constructs.

The Ets2 (thr72) residue is not required for reversion activity. We previously showed that phosphorylation of the Ets2(thr72) residue is essential for the Ras pathway-mediated increase in Ets2 transcription activity. An Ets2 mutant containing a Thr to Ala substitution still showed basal transcriptional activity but lost Ras responsiveness (4,18). To test the connection between Ras signaling to Ets2 and the ability of expressed Ets2 to revert transformation, I determined whether expression of full-length Ets2(A72) or Ets2TAD(A72) could reverse anchorage-independent growth in soft agar. I generated stable lines overexpressing the Ets2(A72) or Ets2TAD(A72) and tested individual lines for their growth in soft agar. Both of the tested constructs clearly caused significant decreases in anchorage-independent growth (fig. 1A), with lines that showed an over 4-fold reduction compared to the lines containing the empty vector, indicating that phosphorylation on this site is not essential for the reversion activity of Ets2. In addition, I analyzed the effects of these mutants on cell morphology and stress fiber formation. Like their wild-type counterparts, full-length Ets2(A72) showed morphological reversion and stress fiber reorganization, but Ets2TAD(A72) did not.

Ets2 constructs act downstream of MAP kinase activation. In order to determine that the overexpression of the Ets2 constructs don't revert the cells to more normal growth, just by extinguishing the expression of Ras, I confirmed Ras expression levels by western blot using the pan-Ras antibody. All of the tested cell lines showed the same Ras expression. I also postulated that the Ets2 mutants would act downstream of the MAP kinases ERK1 and ERK, since they are downstream of Ras/Raf/MEK signaling but still upstream regulators of Ets2 (20). By using an immune complex assay I found that there was no significant change in the basal level of ERK activity in the control lines and the reverted lines, indicating that the Ets2 constructs were not altering ERK activity.

Milestone 4: Effect of the MAPK inhibitor PD098059 on reversing the transformed phenotype of DT-3T3 cells. As a model for small molecule inhibitors of downstream signaling, I tested the effect of the MEK activation inhibitor PD098059 on the RAS transformed fibroblasts. This compound that blocks activation of MEK, was added to the media in 3 different concentrations (10, 30, and 100μM) and the effect on attached

growth and soft agar growth was tested. I found that even in concentrations of 100μM end concentration this compound did not have any effect on attached or soft agar growth of these cells (fig. 2).

Objective 2: Reversion of the transformed phenotype of breast cancer cell lines.

Milestone 5: Establishing breast cancer cell lines expressing Ets2 mutants. The extensive analysis in objective 1 helped me to identify the Ets2 constructs that are potent in reversing the transformed phenotype of the DT-3T3 cells. As a first step I tried to establish the highly invasive MDA-MB-435 cell line overexpressing the Ets2DBD, full-length Ets2, VP16Ets2, or the empty expression vector by lipofectamine transfection. I obtained only about 10% of G418 resistant clones in the transfection with full-length Ets2 compared to the transfection with the dominant inhibitory mutant Ets2DBD or the empty vector control transfection. I was not able to get any resistant colonies using the VP16Ets2 construct. An additional problem arose when I tried to expand these full-length expressing MDA-MB-435 lines. Most of these cell clones started to round up and die, which gave me a very low number in expandable full-length expressing lines. After testing various growth conditions, I found out that MDA-MB-435 conditioned media helped these full-length expressing lines in their growth. Western blot analyses of 6 different full-length Ets2 expressing lines and 7 Ets2DBD lines showed a whole range of protein expression for the Ets2DBD lines (fig 3). The G418 resistant, expandable clones containing the full-length construct showed very low protein expression. Taking these problems into account, I hypothesize that the full-length Ets2 or the even more powerful VP16Ets2 is somewhat toxic to these cells and that it is a fundamental problem is to find the right protein amount that does not kill these cells but is high enough to have any biological effect. To date I have hints that a high amount of full-length expression leads to a high stable apoptosis rate in these cells.
In order to overcome the toxicity of over expression of full-length Ets2, I decided to move to an inducible system. I chose the Tet-On system from Clontech, as this system has successfully been used for other cell lines in our laboratory.

Milestone 6: Expression of full-length Ets2 or the Ets2DBD in MDA-MB-435 cells inhibits anchorage-independent growth in soft agar. Similar to the DT-3T3 cells, I tested the stable MDA-MB-435 lines for their ability to grow in soft agar. In order to exclude clonal variations, I tested 4 control clones containing the empty vector, 8 clones expressing the dominant inhibitor Ets2DBD and 10 full-length Ets2 clones. Four of the full-length clones showed complete inhibition of soft agar colony formation. In addition, two clones showed a drastic reduction (>50%) in colony formation. For the Ets2DBD expressing clones, I observed that 50% of the lines showed a more than 70% reduction in soft agar growth (example shown in fig.4). None of the control clones showed significant reduced growth in soft agar. Western blot analysis of the Ets2DBD lines and the full-length Ets lines showed a correlation between the amount of protein they are expressing and the loss of anchorage independent growth.
The proposed analysis of changes in chemotaxis is under way.

Milestone 7: Effect of PD098059 on reversing the transformed phenotype of MDA-MB-435 cells. Like for the DT-3T3 cells, I tested the effect of PD098059 on attached and soft agar growth of the MDA-MB-435 cells. In contrast to the results for the DT-3T3 cells, PD098059 had profound effects on both, soft agar and attached cell growth. Even the low amount of 10μM of this compound reduced growth of these cells significantly (see fig 5). In order to get some insight into the mechanism how the different Ets2 constructs might lead to loss of anchorage independent growth, I performed transient transfection experiments of full-length Ets2, Ets2DBD or the empty pCIN vector with several Ets-dependent reporter genes. Figure 6 shows that Ets2 can activate E.18, an Ets-dependent reporter gene that contains two Ets binding sites, up to 17.5 fold. The dominant

inhibitor of Ets-dependent transcription, Ets2DBD, clearly inhibited the expression of this reporter gene.

Objective 3: ***In vivo*** **analysis of the reversion of tumorigenicity and metastasis induced by Ets2 mutants.**

Milestone 8: Ets2 expression reduces tumorigenicity of DT-3T3 cells in nude mice. In order to study whether the Ets2-mediated morphological reversion and loss of anchorage independent growth of DT-3T3 cells *in vitro* reflect a reduced ability to form tumors *in vivo*, I performed tumorigenicity assays in nude mice. For this study I injected the parental DT-3T3 cells, clones that are expressing full-length Ets2, the EtsDBD, VP16Ets2, Ets2TAD or lines that carry the empty expression vector in the left and right dorsal flank of 4 weeks old nude mice. Tumor growth was measured externally by caliper and the average tumor volume in mm^3 was determined. The numbers in Table 1 represent the average of 6-8 tumors derived from each cell line. I saw no significant difference in tumor growth for both of the pCIN lines that I tested. In contrast, both of the full-length Ets2 expressing DT-3T3 lines showed a drastic delay in tumor formation. Reduction in tumor growth was also seen in Ets2DBD#10 and Ets2TAD#53, but the tumor volumes were only statistically significant on day 6 and 8 for the Ets2TAD line and only on day 8 for the Ets2DBD line. The parental DT-3T3 cell line and also the control lines showed a very rapid onset of tumor growth, so that at day 10, the tumors were so big, that they started to exhibit reduced growth. At that time, the tumors for the other tested lines were able to catch up, and by day 12 to 14, none of the tumor volumes were different from the ones of the control line pCIN#5. It is likely that the delayed tumor growth of the Ets mutant-expressing lines represent the *in vivo* effect of these Ets proteins and that the absence of G418 in the *in vivo* experiment lead to subsequent selection of cells in which expression from the pCIN vector is reduced. Analysis of a delayed onset tumor in a full-length Ets2-expressing line supported this idea, as expression of the introduced Ets2 was no longer visible. Taken together, the ability of the activating constructs Ets2 or VP16Ets2 to cause substantial delay in tumor formation, and the Ets2DBD to cause a delay in tumorigenicity correlates with their ability to revert the transformed phenotype in *in vitro* experiments.

DT cells transfected with:	Number of stable lines with indicated soft agar colony formation (relative to DT)			
	>50%	25-50%	10-25%	<10%
pCIN	5	0	0	0
Ets2full	2	1	4	4
Ets2DBD	6	1	5	3
EtsTAD	1	0	4	2
E2TADA72	1	2	2	1
E2FullA72	0	2	2	1
VP16-E2full	0	0	0	10
VP16 only	6	1	0	2

Figure 1A:
Soft agar and attached growth of individual stable DT-3T3 clones

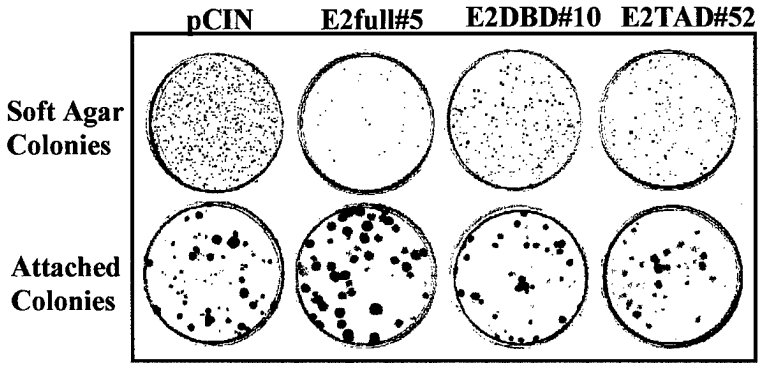

Figure 1B: Percent soft agar colony formation relative to DT-3T3 cells

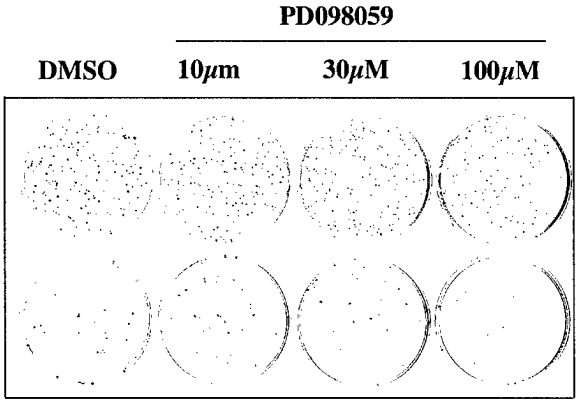

Figure 2: Soft agar and attached colony formation of DT-3T3 cells incubated with the MEK activation inhibitor PD098059

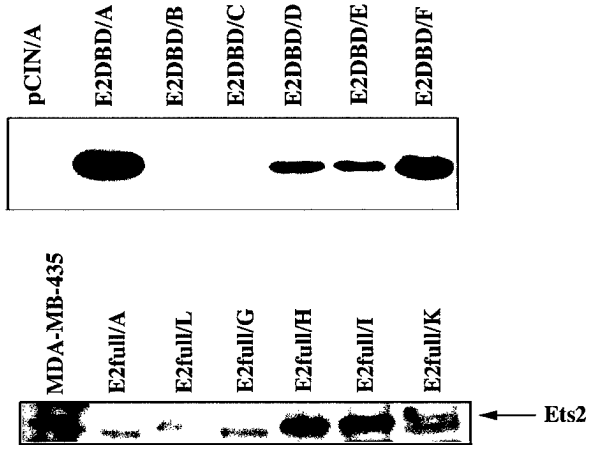

Figure 3:
Expression levels of Ets2 constructs in stable MDA-MB-435 breast cancer lines

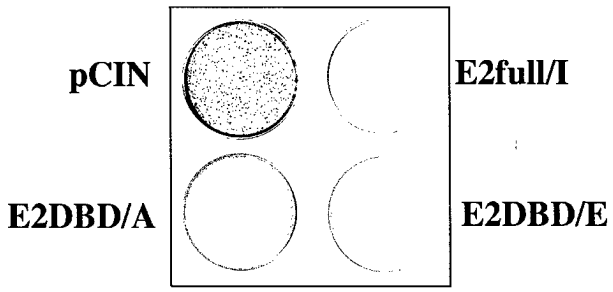

Figure 4: Soft agar colony formation of individual stable MDA-MB-435 clones

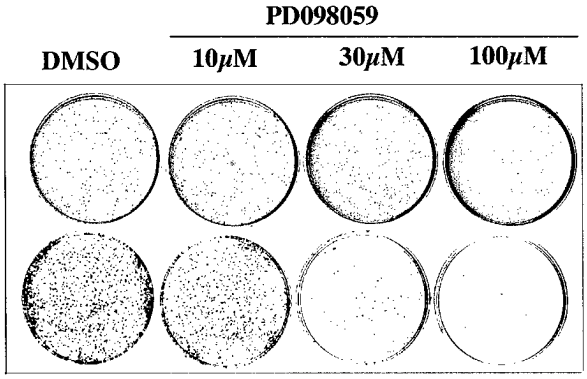

Figure 5: Soft agar and attached growth of MDA-MB-435 cells incubated with PD098059

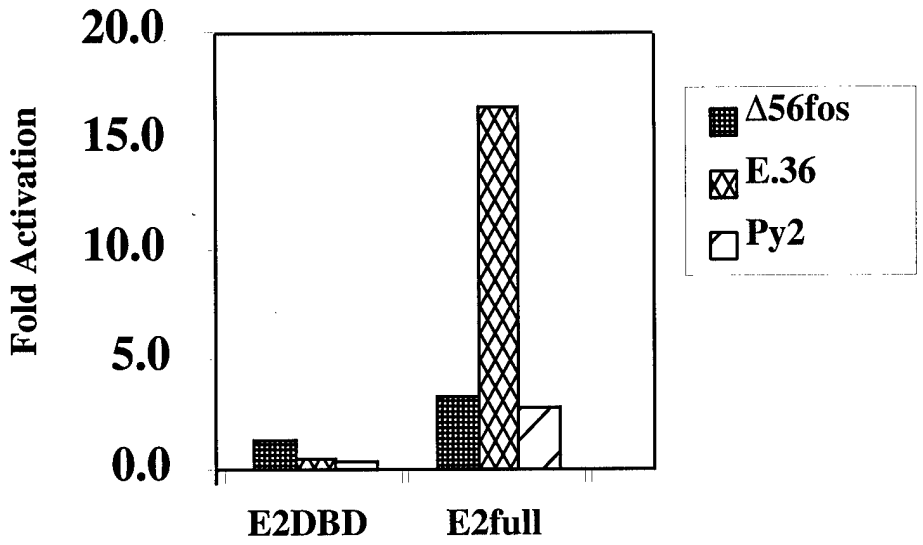

Figure 6: Ets-dependent transcription in MDA-MB-435 cells. MDA-MB-435 mammary tumor cell lines were transfected with either a minimal promoter-containing Δ56Fos-Luc reporter, or this reporter with multiple Ets2 binding sites (E.36) or multiple Ets-AP-1 elements (Py2). Also included was either pCIN4 expression vector, or this vector expressing Ets2DBD or full-length Ets2. Fold activation is the activation of the reporter gene by Ets2 or Ets2DBD compared to pCIN.

Cell Line	Tumor Volume (mm^3)			
	day 4	day 6	day 8	day 10
pCIN5	19±17	263± 159	1,331±649	2,816 ±1,272
full#5	**4**±7	**51** ± 39	**390** ±246	**1,223** ±661
full#6	8±16	**103** ± 62	**599** ±379	**1,467** ±949
DBD#10	16±11	147± 98	**479** ±228	1,736 ±1,138
TAD#53	8±16	**49** ± 51	**357** ±277	1,618 ±1,004
VP16full#1	22±13	**76** ± 62	**630** ±516	**1,553** ±973
VP16full#7	**7**±8	**93** ± 87	**255** ±245	**1,197** ±1,056

Table 1: Tumor formation in nude mice

Summary and Conclusions

During the first 2 years of my fellowship, I achieved considerable progress in all of the proposed Objectives. I showed that elevated expression of the transcriptional activator Ets2 and a dominant inhibitory mutant Ets2DBD can both specifically reverse the transformed phenotype of Ras-transformed NIH3T3 cells. The tested reversion parameter included reduction of anchorage-independent growth in soft agar, reappearance of stress fibers, and delayed tumor formation in nude mice and saturation density.

In addition, I assayed an Ets2 construct that resembles a more specific inhibitor, the Ets2TAD, that does not bind DNA. This construct was able to only partially revert the DT-3T3 cells. The clones tested showed loss of anchorage independent growth, but in contrast to Ets2DBD or full-length Ets2 did not reverse cell morphology. These data suggest that the function of Ets2 on anchorage independent growth and cell morphology are separable. Using an Thr to Ala mutation on position 72 of the Ets2 protein, I demonstrated that this phosphorylation site, which is essential for Ras-mediated increase in Ets2 transactivation activity (4,18), was not essential for reversion activity in all the assays performed.

To further test the effect of dominant inhibitory and activating Ets2 mutants on human breast tumor cell lines, I generated MDA-MB-435 cells that overexpress full-length Ets2 or the Ets2DBD. I did not succeed in establishing lines that express the potent transcriptional activator VP16Ets2. I had to put a lot of effort in generating the full-length Ets2 expressing MDA-MB-435 lines, because Ets2 seems to be quite toxic to these cells. By trying to expand the small G418 resistant colonies, most of them started to die. I have some preliminary data suggesting that a high amount of Ets2 protein in these cells can lead to apoptosis.

It was previously hypothesized that inhibiting Ets dependent transcription by the use of dominant inhibitory Ets mutants, the Ets DNA-binding domains, block the transcription of genes that are responsible for cellular transformation (21). It is therefore somewhat unexpected that expression of full-length Ets2 had strong and broad reversion activity. To investigate whether the activation activity of Ets2 contributes to its reversion activity, I synthetically increased the activation activity by adding the VP16 domain to full-length Ets2. In DT-3T3 cell, this construct showed the strongest reversion activity *in vitro* and *in vivo*, indicating that activation of Ets-dependent transcription can lead to reversion of cellular transformation. Related findings have been published, where overexpression of Ets1 caused partial reversion of the transformed phenotype of DLD-1 colon cancer cells (10) and an Ets1 mutant that had lost its transactivation activity also lost its reversion activity. I did not succeed in establishing MDA-MB-435 cells overexpressing the VP16Ets2 construct, indicating that strong transcriptional activation in these cell line can not be tolerated. Overall these studies show, that altering Ets activity by inhibiting or activating Ets-dependent transcription can reverse Ras-mediated cellular transformation in fibroblasts. In addition I found that cellular transformation in the human breast cancer cell line MDA-MB-435 is dependent on the right balance of Ets factors in these cells. I have used the Ets2 constructs as a tool to explore the role of Ets proteins in cellular transformation.

Key Research Accomplishments

(A) Stable expression of different dominant inhibitory and activating Ets2 constructs in RAS transformed NIH 3T3 cells leads to loss of anchorage independent growth of DT-3T3 cells and a human breast cancer cell line MDA-MB-435.

(B) Reversion activity of the introduced Ets2 constructs is correlated to their expression level in the individual stable clones.

(C) Overexpression of full-length Ets2, the EtsDBD but not Ets2TAD leads to morphological reversion of DT-3T3 cells and to stress fiber reorganization.

(D) The Ets2 (thr72) residue is not required for reversion activity in DT-3T3 cells.

(E) Ets2 constructs act downstream of MAP kinase activation.

(F) The MAPK inhibitor PD098059 has no effect on reversing the transformed phenotype of DT-3T3 cells, but it abolishes soft agar and attached growth of MDA-MB-435 cells.

(G) Ets2 expression reduces tumorigenicity of DT-3T3 cells in nude mice.

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Reportable Outcomes

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